EP

PCT/EP 99/02660

09/673710

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

98201279.1

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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.:
Demande n°: 98201279.1

Anmeldetag:
Date of filing:
Date de dépôt: 21/04/98

Anmelder:
Applicant(s):
Demandeur(s):
CropDesign N.V.
9052 Zwijnaarde 3
BELGIUM

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Stress tolerant plants

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/82, C12N15/29, A01H5/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:
The application was transferred from the original applicant Vlaams Interuniversitair Instituut voor Biotechnologie vzw. t the above-mentioned applicant on 09.09.1998

OAV2/DI/STRESS/001-015/EP

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Stress tolerant plants

The present Invention relates to a method for obtaining stress tolerant plants, for example tolerant to high salt concentrations, to vectors comprising genetic information capable of conferring said tolerance to the plants, to muteins encoded by the said genetic information and to plants and plant materials obtainable by the said method.

Abiotic stress conditions, such as shortage or excess of solar energy, water and nutrients, high and low temperature and pollution (e.g. heavy metals), can have a major impact on plant growth and can significantly reduce the yield of e.g. cultivars In the state of the art it is known that, under conditions of abiotic stress, the growth of plant cells is inhibited by arresting the cell cycle in late G_1, before DNA synthesis, and/or at the G_2/M boundary (See reviews of Dudits et al., 1997, Plant Cell Division, Portland Press Research, Monograph, Francis, D., Dudits, D. and Inzé, D. eds, ch 2, p21 ff., and Bergounioux et al., 1988, Protoplasma, 142, p. 127-136).

The regulation of the cell cycle in plant cells is however poorly understood. WO 92/09685 describes the presence of a $p34^{cdc2}$ protein in plants, a protein which is known to play a key role in the cell cycle of yeasts and vertebrates (see e.g. the review by Lew, D. J. and Kornbluth, S. in Curr. Op. Cell Biol., 1996, 8:795-804, herein incorporated by reference), wherein an indication is made that the amount of plant $p34^{cdc2}$ protein becomes limiting for cell division in plant tissue. However, no clue whatsoever was made related to the role of $p34^{cdc2}$ protein or other putative plant cell cycle control proteins in arresting the cell cycle under conditions of abiotic stress nor related to the onset of cell cycle progress after the said cell cycle arrest.

In investigating the behaviour of plant cells and plants under conditions of abiotic stress, the present inventors have been able to show that stress-dependent downregulation of the cell division (cell cycle) is mediated by endogenous cellular components. Said components may comprise cell cycle regulatory proteins that may undergo stress induced alterations, thereby being activated or deactivated.

Moreover, it could surprisingly be shown that one could confer to the plant the capacity to counteract or even avoid the downregulation of the cell division under conditions of abiotic stress, thus enabling th plant to be tolerant to the said stress conditions, by e.g. altering, or inhibiting, or competing with, or circumventing the regulatory actions of, the

above-mentioned endog nous cellular components.

It was found that in plants, at the onset of the cell cycle arrest at the abiotic stress conditions, the endogenous Cyclin Dependent Kinases (CDK's) were phosphorylated at a tyrosine at position 15 and optionally also at a threonine residue at position 14.

In most plants for which CDK sequences have been identified, the positions of the said tyrosine and threonine residues are at positions 15 and 14, respectively; this is e.g. the case for CDC2a of *Arabidopsis thaliana*. It is however possible that, e.g. during the course of evolution, in some plant CDK's the respective positions of these consensus Y-15 and T-14 have been shifted somewhat, i.e. as a result of one or more deletions or additions at the N-terminus of the protein. The terms "tyrosine at position 15" and "threonine residue at position 14" as used herein, are therefor meant to encompass the positions 14 and 15 of the respective CDK, as well as such positional changes of the said tyrosine and threonine residues within the plant CDK protein, wherein the characteristic of these residues being phosphorylated at the onset of the stress-induced cell cycle arrest is retained. This means that the said positions as defined herein correspond to the tyrosine at position 15 and the threonine at position 14 of CDC2a of *Arabidopsis thaliana*, respectively.

With "plant CDK" is meant to encompass all plant CDK proteins having a cell cycle regulatory function in plants or plant cells having the above-mentioned phosphorylatable tyrosine residue, and optionally in addition thereto, the said threonine residue. Examples of these CDK's are the members of the CDC2 family, as identified in *Arabidopsis thaliana*, such as CDC2a and CDC2b.

In particular it was found that at the onset of the cell cycle arrest at the abiotic stress conditions, the plant CDK protein, being functionally equivalent to the known CDC2a of *Arabidopsis thaliana*, was phosphorylated at a tyrosine and optionally also at a threonine residue, corresponding to the tyrosine of position 15 and the threonine of position 14 of said CDC2a respectively.

The said phosphorylation appeared to be one of the key events in abiotic stress-induced cell cycle arrest.

With "plant CDK protein, functionally equivalent to the known CDC2a of *Arabidopsis thaliana*" is meant each CDK protein having a similar regulatory function as CDC2a of *Arabidopsis thaliana* in plants or plant cells respectively, e.g. having the PSTAIRE cyclin binding motif, and the above-mentioned phosphorylatable tyrosine and threonine residues.

It could surprisingly be shown that the downregulation of the cell division of plants, exposed to abiotic stress, was effectively counteracted by the presence of a CDK, in particular CDK, equivalent to CDC2a of *Arabidopsis thaliana*, being fr e of a phosphate at

the Y-15 position. A preferred embodiment of the present invention therefore relates to conferring to the plant the capacity to provide, at the stress conditions, CDK protein, being functionally equivalent to CDC2a of *Arabidopsis thaliana*, a substantial portion thereof being free of phosphate at the tyrosine, corresponding to the tyrosine of position 15 of said CDC2a. A "substantial portion" in this respect is defined herein as the amount of CDK, being free of phosphate at the Y-15 position, that is sufficient to confer to the plant improved growth during abiotic stress conditions. The person skilled in the art will understand that not all of the corresponding CDK present in the plant or plant cell has to be phosphate free at the Y-15 position to improve said stress tolerance. This may e.g. be accomplished by conferring to the plant the capacity of preventing phosphorylation of the said tyrosine, or of activating the dephosphorylation mechanism for the said tyrosine. As this counteraction may further be improved upon T-14 being additionally free of phosphate, the CDK protein is preferably free of phosphate groups at both the tyrosine and the threonine, corresponding to the tyrosine on position 15 and the threonine on position 14 of said CDC2a, respectively.

An attractive way to obtain stress tolerant plants according to the present invention is therefore by conferring to the plant the capacity to provide at the stress conditions, CDC25 or a functional analogue thereof, capable of dephosphorylating at least the tyrosine at position 15 of the endogenous CDK of the said plant. The dephosporylating activity of CDC25 is described in Lew and Kornbluth, *supra*. By enabling the plant to produce, at the stress conditions, functional CDC25 protein, i.e. capable of dephosphorylating the above-mentioned tyrosine, and optionally also the adjacent threonine of the endogenous CDK, the phosphorylation of CDK as a result of the stress conditions is effectively counteracted.

Another attractive route to obtain stress tolerant plants according to the present invention is by conferring to the plant the capacity to inhibit, at the stress conditions, the expression or activity of at least Wee-kinase or a functional equivalent thereof, thereby inhibiting the endogenous phosphorylation of CDK of the said plant at at least the tyrosine at position 15. Wee-kinase is reviewed in e.g Lew and Kornbluth, *supra*. This kinase phosphorylates the above-discussed Y-15 of CDK and may also be responsible for the phosphorylation of the T-14. With "functional equivalent of Wee-kinase" is meant any endogenous kinase of the plant having the function of known Wee-kinase in phosphorylating the respective tyrosine residue and optionally the threonine residue of the endogeneous plant CDK. The recently identified Myt1 kinase (Mueller, P. et al., 1995, Science 270, pp 86 ff.) may therefore be regarded as such a functional equivalent. By inhibiting the expression of the Wee-kinase at abiotic stress conditions, the phosphorylation of CDK will be inhibited, reducing the downregulation of th cell cycle arrest, thus

obtaining stress tolerance.

A preferred embodiment of the present invention is by conferring to the plant the capacity to produce, at the stress conditions, a CDK mutein, of which Y-15 is substituted to a non phosphorylatable residue. When the plant is able to produce such a CDK mutein, said mutein will substantially not be sensitive for the phosphorylation system, triggering the stress-induced cell cycle arrest. In this way, the plant circumvents the downregulation of the cell cycle, being more tolerant to said stress conditions. The term "CDK mutein", used herein, is defined as a CDK fragment or CDK protein comprising at least one mutation, e.g. an amino acid substitution, deletion or addition. Additionally, phosphorylation of T-14 may play a potentiating or mediating role in the above-discussed downregulation mechanism. Therefore, in another preferred embodiment of the method according to the present invention, the CDK mutein also comprises a non phosphorylatable amino acid residue at position 14.

Preferably, the said mutein is derived from endogenous CDK of the stress tolerant plant to be obtained. By starting from the endogeneous CDK, the risk of malfunctioning muteins is minimised. However, in view of homology between different plant CDKs, it will be obvious to the skilled person that it is also possible to use CDK from another plant species. CDK, of e.g. yeast or verterbrate origin may, dependent on the homology with the endogenous plant CDK, as well be suitable in the present invention; the suitability can easily be determined by the skilled person.

In a preferred method for obtaining stress tolerant plants according to the present invention, the said capacity is conferred to one or more cells of said plant by a) transforming one or more plant cells with a vector, at least comprising, under the control of a promoter functional in the said plant cells, a DNA sequence, coding for a mutated *cdk* gene of *Arabidopsis thaliana* or functional equivalent gene of another species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising a non phosphorylatable amino acid residue at the position of the CDK mutein, corresponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*, b) by regenerating a plant from one or more of the transformed plant cells, e.g. by the *Agrobacterium tumefaciens* transformation system. However, other transformation methods known in the field may be used. With "mutein, functional in plant cells", muteins are meant, which, when expressed in the said plant cells, lead to improved stress tolerance of the said cells.

Preferably, the mutein also comprises a non phosphorylatable amino acid residue at position 14 of the CDK mutein.

As non phosphorylatable amino acid residue substituting Y-15 (i.e. the tyrosine of th CDK, corr sponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*), th CDK mut in preferably comprises a Y-15 -> F-15 mutation, F being phenylalanine. In

all plants investigated so far, the expression of said mutein led to enhanced stress tolerance.

Similarly, as non phosphorylatable amino acid residue substituting T-14 (i.e. the threonine of the CDK, corresponding to the threonine on position 14 of CDC2a of *Arabidopsis thaliana*), the CDK mutein preferably comprises a T-14 -> A-14 mutation, A being alanine. Expression of such a mutein led to even improved stress tolerance.

Any promoter that functions in the target cells can be used. The use of the CaMV35S promoter, per se known to the skilled person, resulted in plants with improved tolerance to salt stress (i.e. to a salt concentration in the growth medium of e.g. 1 w/v % NaCl). It is preferable to use a promoter, that can be induced upon the abiotic stress conditions; such promoters are known in the art (see e.g. Lang, V. and Palva, E. T., 1992, Plant Mol. Biol., 20, p 951 ff.).

It is to be understood that the skilled person, aware of the above teaching, will be able to apply numerous techniques to confer to a plant the capacity to counteract the stress-induced downregulation of cell cycle progress as is discussed above. Instead of or in addition to transforming plant cells with a gene coding for a CDK mutein, it is possible to overexpress in the plant cells CDC25 or functional analogue thereof by transforming the said cells with a functional *cdc25* gene under the control of a suitable promoter, e.g. the CaMV35S promoter, or e.g. to transform the plants with nucleic acids coding for anti-sense RNA, capable to basepair with, and leading to cleavage of, the mRNA coding for any protein that is desired to be knocked out, like Wee-kinase-mRNA.

The present invention also relates to a vector, at least comprising a promoter, functional in plant cells, operably linked to a DNA sequence, coding for a mutated *cdc2a* gene of *Arabidopsis thaliana* or functionally equivalent gene of another species, preferably a plant species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising, in the CDK mutein, a non phosphorylatable amino acid residue at the position, corresponding to the tyrosine on position 15 of CDC2a. Preferably, the mutein also comprises a non phosphorylatable amino acid residue at the position of the mutein, corresponding to the threonine on position 14 of said CDC2a. In order to minimize the possibility of malfunctioning, it is preferred that a plant CDK gene is used. Being transformed with a vector of this type, plant cells are capable of producing, at abiotic stress conditions, CDK muteins that are not susceptible to the above-discussed regulatory phosphorylation events, therefor leading to stress tolerant plants or plant cells.

In a further embodiment, the vector comprises a promoter, functional in plant cells, operably linked to a DNA sequence, coding for CDC25 or a functional analogue thereof, capabl of dephosphorylating at least the tyrosin of at least one plant CDK, corresponding with the tyrosin on position 15 of CDC2a of *Arabidopsis thaliana*. Such a

vector can be used to transform plants in order to, as is discuss d above, express CDC25 in plants, resulting in dephosphorylation of Y-15 and optionally the T-14 of the endogenous plant CDK, l ading to improved stress tolerance.

The invention will be further illustrated with the following non-limiting examples.

Example 1.

Morphological alterations in response to salt stress and correlation to the expression of cell cycle regulatory genes.

In order to investigate morphological alterations in response to salt stress, an histochemical analysis of three known plant cell cycle regulating proteins was performed. Therefor, time course experiments were performed on transgenic plants transformed with cyclin (CycA2;1, CycB1;1) and CDK (CDC2aAt) promoter-gus fusions respectively. Both cyclin and CDK promoters originated from *Arabidopsis thaliana*. Ten days old Arabidopsis plants were transferred to solid media containing 1% NaCl ; GUS activity and morphological changes were observed after 12hrs, 36hrs, 4 days, 1 week and two weeks. After 12hrs treatment the promoter activities declined in the apical meristem, before any morphological change was visible. After 36 hrs growth in the presence of salt the swollen roottips showed a decrease in expression of all cell cycle genes concomitant with a shrinkage of the root apical meristem. After four days, morphological alterations in the aerial part of the stressed plant were clearly visible when compared to control. The stressed plants were shorter due to a less elongated hypocotyl and the leaves were smaller. During adaptation to stress an induction of CycA2;1 and CycB1;1 expression in the shoot apical meristem could be noticed. Measurements of the length of the leaves and of the length of the meristematic region in the roots made after two weeks growth on salt containing medium, demonstrated a strong reduction in comparison to control plants. The number of leaves initiated was significantly lower in salt stressed plants than in control plants. In expanding leaves of salt stressed plants no GUS staining for CycB1;1 nor CDC2aAt expression could be detected contrarily to control plants, illustrating the decline in mitotic activity in these organs.

Example 2.

Improved tolerance to salt stress of Arabidopsis thaliana containing a CDC2a-Y15F/T14A mutant gene under the control of a CaMV35S promoter

A comparative study was made between transgenic Arabidopsis plants containing a CDC2a-Y15F/T14A mutant gene und r the control of a CaMV35S promoter, and wild-typ plants, in response to salt stress. Arabidopsis plants (ecotype C24) were engineered containing a mutated form of the CDC2aAt gene in which the phosphorylation sites T14

(Threonine at amino acid position number 14) and the Y15 (Tyrosine at amino acid position number 15) were changed in A14 (Alanine at amino acid position number 14) and F15 (Phenylalanine at amino acid position number 15) under control of a CaMV35S promoter. The overexpression of mutant CDC2a-Y15F/T14A in Arabidopsis lines did not show drastic changes in development. Only a tendency for loss for apical dominance could be noticed. Two CDC2a-Y15F/T14A mutant lines of Arabidopsis were selected to study their response on salt stress. As controls non-transformed Arabidopsis plants (C24) and transgenic plants carrying a construct of a non-mutated CDC2aAt gene under the control of a CaMV35S promoter were included in the experiment. Plants of ten days old, grown on solid germination medium, were transferred to the same medium containing 1% NaCl and their further growth and development was observed compared to the control plants. Both mutant lines displayed an improved tolerance to salinity which was phenotypically visible. The salt stressed mutant plants had bigger and more elongated leaves than control non transformed plants and plants overexpressing the wild type CDC2aAt gene.

CLAIMS

1. Method for obtaining plants, tolerant to abiotic stress conditions, by conferring to a plant the capacity to counteract the stress-induced downregulation of cell division, mediated by endogenous cellular components.

2. Method according to claim 1, characterised by conferring to the plant the capacity to provide, at the stress conditions, Cyclin Dependent Kinase (CDK) protein, a substantial portion thereof being free of phosphate at the tyrosine at position 15.

3. Method according to claim 2, the CDK protein being free of phosphate groups at both the tyrosine and the threonine, corresponding to the tyrosine at position 15 and the threonine at position 14, respectively.

4. Method according to claim 2 or 3, characterised in that the Cyclin Dependent Kinase (CDK) protein is functionally equivalent to CDC2a of *Arabidopsis thaliana*.

5. Method according to any of the preceding claims, characterised by conferring to the plant the capacity to provide at the stress conditions, CDC25 or a functional analogue thereof, capable of dephosphorylating at least the tyrosine at position 15 of the endogenous CDK of the said plant.

6 Method according to any of the preceding claims, characterised by conferring to the plant the capacity to inhibit, at the stress conditions, the expression or activity of at least Wee-kinase or a functional equivalent thereof, thereby inhibiting the endogenous phosphorylation of at least the tyrosine at position 15 of the CDK of the said plant.

7. Method according to any of the preceding claims, characterised by conferring to the plant the capacity to produce, at the stress conditions, a CDK mutein, of which the tyrosine at position 15 is substituted to a non phosphorylatable residue.

8. Method according to claim 7, characterised in that the CDK mutein also comprises a non phosphorylatable amino acid residue at position 14.

9. Method according to any of th preceding claims, characterised in that th said capacity is conferred to one or more cells of said plant by a) transforming one or

more plant cells with a vector, at least comprising, under th control of a promoter functional in the said plant cells, a DNA s quence, coding for a mutated *cdk* gene of *Arabidopsis thaliana* or functional equivalent gene of another species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising a non phosphorylatable amino acid residue at the position of the CDK mutein, corresponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*, b) by regenerating a plant from one or more of the transformed cells.

10. Method according to claim 9, characterised in that the mutein also comprises a non phosphorylatable amino acid residiue at the position of the CDK mutein, corresponding to the threonine on position 14 of said CDC2a.

11. Method according to claim 9 or 10, characterised in that the CDK mutein comprises a Y-15 -> F-15 mutation.

12. Method accordiing to claim 10 or 11, characterised in that the CDK mutein also comprises a T-14 -> A-14 mutation.

13. Method according to any of the claims 9-12, characterised in that the *cdk* gene is under the control of a promoter, inducable by abiotic stress conditions.

14. Vector, at least comprising a promoter, functional in plant cells, operably linked to a DNA sequence, coding for a mutated *cdc2a* gene of *Arabidopsis thaliana* or functionally equivalent gene of another species, preferably a plant species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising a non phosphorylatable amino acid residue at the position of the CDK mutein, corresponding to the tyrosine on position 15 of CDC2a.

15. Vector according to claim 14, characterised in that the mutein also comprises a non phosphorylatable amino acid residue at the position of the mutein, corresponding to the threonine on position 14 of said CDC2a.

16. Vector according to any of the claims 14-15, characterised in that the mutated *cdk* gene sequence comprises the coding sequence for CDC2a of *Arabidopsis thaliana*, Y-15 being mutated to F-15.

17. Vector according to any of the claims 14-15, characteris d in that the mutated *cdk* gene sequence comprises the coding sequence for CDC2a of *Arabidopsis thaliana*, Y-15 being mutated to F-15 and T-14 to A-14.

18. Vector, at least comprising a promoter, functional in plant cells, operably linked to a DNA sequence, coding for CDC25 or a functional analogue thereof, capable of dephosphorylating at least the tyrosine at position 15 of at least one plant CDK.

19. Vector according to any of the claims 14-18, characterised in that the promoter is inducible by abiotic stress conditions.

20. Plant cell, transformed with a vector according to any of the claims 14-19.

21. Plant, tolerant to abiotic stress, in particular salt stress, obtainable by the method according to any of the claims 1-13.

22. Progeny of a plant according to claim 21.

23. Plant material such as roots, flowers, fruit, leaves, pollen, seeds, seedlings or tubers, obtainable from a plant according to claim 21 or 22.

24. Mutein of CDK, functionally equivalent to CDC2a of *Arabidopsis thaliana*, the mutein being functional in at least one plant species, comprising a non phosphorylatable amino acid residue at the position of the CDK mutein, corresponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*.

25. Mutein according to claim 24, characterised in that the mutein also comprises a non phosphorylatable amino acid residiue at the position of the mutein, corresponding to the threonine on position 14 of said CDC2a.

ABSTRACT

A method for obtaining plants, tolerant to abiotic stress conditions, in particular salt stress, is described, by conferring to a plant the capacity to counteract the stress-induced downregulation of cell division mediated by endogeneous cellular components. Also vectors for conferring the said capacity are enclosed.